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(57) Abstract

Monoclonal antibodies inhibiting binding of soluble IgE to human lymphocytes, to monoclonal antibodies to human lymphocyte IgE receptors, and to hybridomas producing such antibodies. These antibodies are preferably of the immunoglobulin subclass IgG_1. They can be used in assaying soluble $Fc_\varepsilon R_{II}$ and cells carrying these $Fc_\varepsilon R_{II}$ receptors. The invention further provides kits for assaying soluble $Fc_\varepsilon R_{II}$ and also cells carrying these $Fc_\varepsilon R_{II}$ receptors by means of monoclonal antibodies described herein.

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MONOCLONAL ANTIBODIES TO HUMAN LYMPHOCYTE IgE RECEPTORS, HYBRIDOMAS PRODUCING SUCH ANTIBODIES, AND KITS FOR USING SUCH ANTIBODIES

I. INTRODUCTION

This invention relates to novel monoclonal antibodies to the human lymphocyte IgE receptors, that is, to monoclonal antibodies directed at the receptor for the Fc fragment of human IgE which is present in lymphoid cells ($Fc_\varepsilon R_{II}$), to hybridomas producing such antibodies, to kits for using such antibodies, e.g. in assaying or detecting these receptors on cells, and to related soluble molecular entities of the IgE binding factor type (=IgE BF).

It is well known in the art that it is possible to obtain a cell line which is able to produce a homogenous, i.e. monoclonal, antibody. The basic technique (Kohler and Milstein, Nature 256, 1975) comprises the fusion of mouse myeloma cells with spleen cells to form hybridoma cells and selection from these of clones capable of producing the desired antibody. This general procedure has also been described in U.S. Patents 4,364,932, 4,364,934, 4,364,935, 4,364,937 and 4,361,550.

Although the general method has been known for some years, the preparation and selection of each suitable hybridoma presents its own special

difficulties. There is indeed no certainty that a suitable hybridoma will be found and, equally, there is no certainty that the hybridoma will produce an antibody having the desired properties.

Monoclonal antibodies have a variety of uses, in particular for the isolation and purification of the proteins to which they are specific or for assaying them e.g. in a diagnostic kit; see for example PCT published applications WO81/02899 and WO82/01773.

The receptor for the Fc fragment of Immunoglobulin E ($Fc_\varepsilon R_{II}$) is a glycoprotein expressed on the surface of certain human cells. It has the capacity of binding IgE and therefore can be considered an IgE binding factor. Suppressive IgE BFs would be of considerable interest in the therapy of diseases linked to elevated IgE levels, such as allergic rhinitis, atopic dermatitis and asthma. IgE potentiating factors, on the other hand, would have utility in the treatment of conditions where increased IgE levels might be required (e.g., elimination of parasites). Assays for such factors exist, but the availability of monoclonal antibodies (Mabs) would greatly help in designing highly sensitive and specific immunochemical assays.

It is therefore an object of the present invention to provide the following:

Monoclonal antibodies inhibiting binding of soluble IgE to human lymphocytes;

Monoclonal antibodies to the human lymphocyte IgE receptors;

Methods for their preparation;

Hybridomas producing them;

Assays using them (for detecting them on human cells);

Kits using them for detection of soluble forms (e.g., in body fluids or culture supernatants).

The invention therefore provides monoclonal antibodies inhibiting binding of soluble IgE to human lymphocytes. The invention further provides monoclonal antibodies to the human lymphocyte IgE receptors. These monoclonal antibodies are preferably of the immunoglobulin subclass IgC_1. The invention further provides monoclonal hybridomas capable of producing these monoclonal antibodies.

According to the invention, monoclonal antibodies inhibit the binding of soluble IgE to human lymphocytes, and are identical or similar to monoclonal antibodies produced from hybridomas obtained according to the following successive steps :

a) The culture of hybridomas is obtained, using several separate containers, by fusion of mice myeloma cells with spleen cells obtained from mice immunized by injections of cells which strongly express IgE receptor, e.g. RPMI 8866, preferably in a medium eliminating unfused myeloma cells and unfused spleen cells, e.g. in a HA medium;

b) the supernatants of the hybridoma cells of each container are tested for the presence of antibodies inhibiting the binding of IgE to the said cells;

c) hybridomas producing antibodies inhibiting the binding of IgE to the said cells are selected;

d) these selected hybridomas are cloned, preferably by the limiting dilution technique, these clones being chosen so as to obtain the desired monoclonal antibodies by culturing the hybridomas.

These monoclonal antibodies are preferably of the immunoglobulin subclass IgG_1.

The aforesaid antibodies are the monoclonal antibody Mab 25 produced by the hybridoma 9P25 A 3/13

deposited at the Institut Pasteur under N° I-548, and the monoclonal antibody Mab 135 produced by the hybridoma 9P 135 D 6/5 deposited at the Institut Pasteur under N° I-549.

The assays for soluble IgE BFs can be of two types, a competition assay or a double sandwich assay (which requires two Mabs binding to different epitopes of the relevant antigen).

In the competition assay, a human IgE binding factor (IgE BF) labelled e.g. with an enzyme (for example E. coli ß-galactosidase) or a radioisotope competes with a non-labelled one (present in the sample to be assayed or a standard) for binding to a Mab linked to a solid support. This requires only one Mab.

In order to produce Mabs specific for human IgE binding factors, mice were immunized with cells expressing on their surface such molecules at high density. Alternatively, mice could be immunized with soluble IgE binding factors.

II. ASSAY FOR HUMAN $Fc_\epsilon R_{II}$ AND CHOICE OF THE CELL LINE EXPRESSING $Fc_\epsilon R_{II}$ AT HIGH DENSITY.

INTRODUCTION

The human cell line RPMI 8866 is known to express $Fc_\epsilon R_{II}$ at relatively high density. An assay has been established which permits detection of $Fc_\epsilon R_{II}$. Briefly, cells to be assayed are successively incubated with IgE, a monoclonal (or polyclonal)anti-IgE antibody, and fluorescent microspheres coupled with anti-mouse (or rabbit) immunoglobulin antibody. The percentage of cells

binding to the fluorescent microspheres (corresponding to the percentage of cells expressing $Fc_\epsilon R_{II}$) is determined by flow cytometry. Details of this are given in a separate section.

This assay was found suitable for the detection of human IgE BF: Preincubation of IgE with IgE BF results in a decreased binding of IgE to its receptor so that a decreased percentage of cells bind to the fluorescent microspheres. This work demonstrated that RPMI 8866 was the best cell line for immunizing mice since it carried much $Fc_\epsilon R_{II}$.

Mabs against $Fc_\epsilon R_{II}$ can be screened for their ability to inhibit the binding of IgE to RPMI 8866 cells. Cells are first incubated with the Mab to be assayed and then IgE is added. The other steps are carried out as described above.

FLOW CYTOMETRIC METHOD FOR DETECTION OF Fc_ϵ RECEPTORS AND IgE BINDING FACTORS USING FLUORESCENT MICROSPHERES

MATERIALS AND METHODS

Antibodies and proteins:

Human myelomatous IgE was purified by ion exchange chromatography (DEAE 52) using a NaCl gradient (0.01M - 0.3M, pH 7.2). According to SDS-PAGE and silver staining, the IgE was pure and uncontaminated by other proteins.

To detect IgE, a monoclonal anti-IgE (Hybritech Inc., La Jolla, CA) and a polyclonal anti-IgE were used. The polyclonal antiserum was prepared by immunizing rabbits with purified IgE PS, and the antiserum was rendered monospecific for IgE by passage over IgG, IgA and IgM immunoabsorbant columns (Affigel-10 Biorad, Richmond, CA).

A rabbit anti-mouse Ig antiserum and a goat anti-rabbit Iq antiserum have been prepared in our laboratory. Their IgG fractions were isolated by column chromatograpy using Trisacryl GF 05 and DEAE Trisacryl (Industrie Biologique Francaise, Genevilliers, France). Human IgG, IgA and IgM were purified from myeloma or Waldenstrom sera using ion exchange chromatography according to standard procedures.

CELLS:

WIL2WT (an EBV (= Epstein-Barr Virus) lymphoblastoid cell line), Daudi (a Burkitt lymphoma), Molt 4 (a T leukemia), HL60 (a promyelomonocytic leukemia) and U937 (a promonocytic cell line) were obtained from the ATCC (American Type Culture Collection; Rockville, Maryland). RPMI 8866 (an EBV lymphoblastoid cell line) was kindly provided by Dr. K. Ishizaka. UD38 is an EBV lymphoblastoid cell line which has been established in our laboratory by Dr. F. Rousset after infection of normal peripheral blood B cells with EBV. All cells were cultured in RPMI 1640 (Flow, Irvine, Scotland) supplemented with 10% heat inactivated fetal calf serum (Flow), 2mM glutamine (Flow), 100 U per ml penicillin and 100 µg per ml streptomycin (Flow).

Coupling of proteins to microspheres

0.1 mg of the protein to be coupled is dissolved in 1 ml of a solution of EDAC (N-(3-dimethyl-aminopropyl)-N'-ethyl-carbodiimide hydrochloride) (0.1 mg per ml distilled water).

100 µl sonicated (1 min in a Bransonic 321) microspheres (Fluoresbrite carboxylate diameter 0.57 u, Polyscience, Warrington, PA, ref 15700) are added. The suspension is incubated overnight at 4°C on a rocking table. After this incubation, the microspheres are

centrifuged (10000 g, 5 min) and washed three times with PBS-BSA (phosphate-buffered saline containing 1% bovine serum albumin). The microspheres are finally kept at 4°C in PBS-BSA supplemented with 15 mM sodium azide. The suspension is sonicated for 1 minute before each experiment.

Staining protocol

Cells are adjusted to 10^7/ml in PBS-BSA. 5 x 10^5 cells (50 µl) are distributed per well of 96 conical wells microtiter plates (Flow, Reference number: 7632105.)

The cells are first incubated 45 min with monomeric IgE (0.5 to 2 µg per 5 x 10^5 cells). Cells are then washed with PBS-BSA. A polyclonal anti-IgE antibody appropriately diluted (e.g., 1/2000) is added. After 45 min, the cells are washed with PBS-BSA. The anti-rabbit Ig microspheres (dilution 1/50) are added.

This cell suspension is layered on top of 3 ml heat-inactivated and filtered FCS in conical tubes (Falcon Ref 2099). After centrifugation at 100 X g for 15 min, the supernatant is slowly aspirated and PBS-BSA (150 µl/well) is added to the cell samples which, after resuspension, are analysed by flow cytometry. All steps are carried out at 4°C.

Fluorescence analysis

Fluorescence analysis was performed with a flow cytometer, FACS 440 (Becton Dickinson, Sunnyvale, CA), equipped with a 5 W argon laser running at 488 nm, 0.5 W. Fluorescence parameters were collected using the built-in logarithmic amplifier after gating on the combination of forward light scatter (FLS) and perpendicular light scatter (PLS) which was used to distinguish viable cells from non-viable ones.

Data were stored in list mode and analysed with a PDP 11/23.

III. IMMUNIZATION OF MICE AND PRODUCTION OF HYBRIDOMAS SECRETING $Fc_{\varepsilon}R_{II}$-SPECIFIC MABS

1. Mice are immunized three times with 50 x 10^6 RPMI 8866 cells injected intraperitoneally in phosphate-buffered saline (PBS). The type of mouse used should not be critical but good results are achieved with BALB/c females. The number of injections and the quantity of cells administered must be such that useful quantities of suitably primed splenocytes are produced. The serum of properly immunized mice inhibits the binding of IgE to RPMI 8866 cells.

2. The immunized mice are killed, their spleens are removed and spleen suspensions are prepared. This procedure follows well-known techniques.

3. The spleen cells are fused with mouse myeloma cells. The technique for fusing myeloma cells with spleen cells is well known. Most preferably the fusion is achieved by warming a mixture of the two cell types with an appropriate fusion promoter, e.g. polyethylene-glycol (PEG) having an average molecular weight from about 1000 to about 4000 (PEG1000). Several mouse myeloma cell lines are known and easily available. Preferred are cell lines which are HGPRT-deficient (HGPRT = Hypoxanthine Guanosyl Phosphoribosyl Transferase) and accordingly will not survive in HA (culture medium comprising hypoxanthine and azaserine). Preferably the myeloma cell line used should be of the non-secreting type in that it does not itself produce any antibody. A suitable cell line for the purpose of this invention is the so-called NS1 cell line. These cells were derived from P3/X63-A8 myeloma cells by Kohler and Milstein.

4. The fused spleen cells are cultured in several separate containers (e.g. in 24-well plates) according to standard procedures. The cell cultures obtained in step 3 are mixtures of fused spleen cells (hybridoma cells), unfused spleen cells and unfused myeloma cells. Preferably the cultivation is carried out in a medium which will eliminate the unfused myeloma cell line, e.g. in an HA medium. Those unfused spleen cells which are non-malignant will normally die after a short period of time, whereas the fused cells, which are $HGPRT^+$, can grow in HA medium.

5. The supernatants of the hybridoma cells in each container are tested for the presence of anti-$Fc_\varepsilon R_{II}$ antibodies. The hybridomas are screened for their ability to inhibit the binding of IgE to RPMI 8866 cells. This test may conveniently be carried out by means of the bead assay described above.

6. Hybridomas producing the desired antibodies are selected and then are cloned preferably by the limiting dilution technique.

7. The desired antibodies are produced by means of the selected hybridomas. This production may be achieved in vitro by culturing the hybridoma in a suitable medium followed by isolation of the antibody, but this method may not yield sufficient quantities. For producing larger quantities of the antibody, an in vivo method is preferably used. The hybridoma is injected back into mice where it will cause production of ascites fluid containing substantial quantities of the desired antibody, which is then isolated according to standard procedures.

IV. CHARACTERISTICS OF THE PRODUCED HYBRIDOMAS

1. Inhibition of IgE binding

Two hybridomas, 9P25 and 9P135 D6/5, which gave two clones 25 and 135 D6/5 rèspectively, have been selected since they produce Mabs (called Mab 25 and Mab 135 respectively) that inhibit the binding of IgE to RPMI 8866 cells; see Table I:

TABLE I

Inhibition of IgE Binding by Mab 25 and Mab 135 Antibodies on the RPMI 8866 Cell Line

c-	c+	Mab 25	Mab 135
8	82	29 (71.6)	56.3 (34.7)

Values are given as percentages. Unbracketed figure gives percentage of positive cells and bracketed figure gives percentage inhibition.

Key:

c-: negative control obtained by incubation of RPMI 8866 cells with polyclonal anti-IgE antibody (1/1000) and anti-rabbit Ig-coated microspheres.

c+: positive control obtained by incubation of RPMI 8866 cells with 1 µg IgE, polyclonal anti-IgE antibody and anti-rabbit Ig-coated microspheres.

Hybridomas 9P25 and 9P135 D6/5 have been deposited at the Institute Pasteur, Paris, France under the numbers I 568 and I 549 respectively.

2. Binding pattern to cell lines

a) Technical aspects

Cells lines are maintained in the standard culture medium RPMI 1640 described above.

Cytofluorographic analysis of monoclonal antibodies binding to cell lines was performed by indirect immunofluorescense with fluorescein-conjugated goat anti-mouse immunoglobulins (G/M FITC) (Grub Laboratories, Vienna, Austria) utilizing a FACS 440. In brief, 5 x 10^5 cells were treated with 0.15 ml of the Mab (diluted or undiluted), incubated at 4°C for 30 minutes, and washed twice. The cells were then reacted with 0.15 ml of a 1:40 dilution G/M FITC at 4°C for 30 minutes, centrifuged, and washed three times. Cells were then analyzed on the FACS 440, and the intensity of fluorescence per cell was recorded on a pulse-height analyzer. Background staining was obtained by substituting a 0.15 ml aliquot of 1:500 ascites from a mouse intraperitoneally injected with a non-producing hybrid clone.

b) Mab 25 (Table II)

It does not bind to Molt 4 cells (a T cell leukemic line); Daudi (a Burkitt lymphoma) and a wide number of different IL_2-dependent human T cell clones (although some of them are found weakly positive). It binds to EBV infected lymphoblastoid B cell lines. It binds weakly to the myelomonocytic cell line U937. The binding of this antibody to the cell lines correlates very well with their $Fc_\varepsilon R_{II}$ expression.

TABLE II

Phenotypic expression of Mabs anti $Fc_\varepsilon R$ on different human cell lines

	*$Fc_\varepsilon R_{II}$	Mab 25	Mab 135	**Mab Anti-DR	Control
U 937	±	16.4	13.4	95.0	1.4
Molt 4	-	0.8	0.6	1.2	0.2
DAUDI	-	2.1	96.4	99.2	0.9
RPMI 8866	+	94.1	91.8	99.8	1.1
MO 14	+	97.8	98.0	98.3	4.8
BME	+	97.9	89.5	99.5	10.4

*Determined by microsphere (bead) assay described above.
**Clone L243 obtained from Becton Dickinson. DR antigens are histocompatibility antigens of Class II.

c) Mab 135 (Table II).

It does not bind to Molt 4 cells but does bind to Daudi cells as well as to all the IL_2-dependent $Fc_\varepsilon R_{II}$-negative T cell clones that have been tested. It also binds to all the EBV infected lymphoblastoid cell lines which have been tested and to the EBV negative B cell lines MO14 and BME. It binds weakly to U937.

In summary, Mab 135 is able to prevent binding of IgE to $Fc_\varepsilon R_{II}$ positive cells; and it also recognizes antigenic determinants which are present on $Fc_\varepsilon R_{II}$ negative cells.

3. Binding pattern to human blood, tonsil and spleen cells

a) Technical aspects

Blood, tonsil and spleen mononuclear cells are isolated according to standard procedures. Tonsils were obtained at tonsillectomy from children with chronic tonsillitis and spleens were obtained from operative specimens of trauma patients. Spleens and tonsils were made into single cell suspensions (in PBS) by being pressed through wire mesh. These cell suspensions as well as diluted blood (in PBS) were next layered over Ficoll-Hypaque and centrifuged (following the technique of Boyum, Scand. J. Clin. Lab. Invest. 21 (supp. 97): 77, 1968). Mononuclear cells were harvested from the Ficoll interface and washed with PBS.

Cytofluorographic analysis of monoclonal antibodies with all cell populations was performed by indirect immunofluorescence as described earlier. Double fluorescence analysis was also performed in order to determine to which cell subpopulation the Mabs bind. The experimental protocol is described in Table III.

Data are summarized in Tables IV, V and VI.

TABLE III

PROCEDURE FOR DOUBLE STAINING CELLS WITH TWO MONOCLONAL ANTIBODIES

1. Adjust cells at 10^7/ml in PBS, 1% BSA, 0.01% Azide.

2. Add the first monoclonal antibody at the appropriate concentration to 40 µl of the cell suspension (in microtiter tray wells).

3. Incubate 15 minutes at 4°C.

4. Wash twice with PBS, 1% BSA, 0.01% Azide.

5. Resuspend cells in 100 µl of a 1/150 dilution in PBS, FITC conjugated $F(ab')_2$ fragments goat anti-mouse immunoglobulins (Grub, Vienna, Austria).

6. Incubate 15 to 30 minutes at 4°C.

7. Wash twice in PBS-BSA-Azide.

8. Resuspend cells in 100 µl PBS-BSA containing 1% mouse serum.

9. Incubate 20 minutes at 4°C and wash twice.

10. Resuspend cells in 40 µl of PBS-BSA.

11. Add the second monoclonal antibody conjugated with biotin.

12. Incubate 15 minutes at 4°C.

13. Wash twice with PBS-BSA-Azide.

14. Resuspend cells in 40 µl PBS-BSA-Azide and add Avidin conjugated to Phycoerythrin (Becton Dickinson).

15. Wash twice and resuspend cells in 250 µl PBS-BSA.

16. Analyze with FACS.

In Tables IV - VI:

- indicates negatively reacting cells (Mab unbound);

+ indicates positively reacting cells (Mab fixed).

Leu 1 is a Mab commercialized by Becton Dickinson which specifically binds to T cells.

B1 is a Mab commercialized by Coulter Immunology which specifically binds to B cells.

Anti-DR is the Mab defined in Table II.

Leu M3 is a Mab commercialized by Becton Dickinson which specifically binds to monocytes.

TABLE IV

PERCENTAGE OF SPLEEN CELLS BOUND BY MABS

SPLEEN*			Mab 25		Mab 135	
			-	+	-	+
TOTAL CELLS			4.8		52.2	
Leu 1	(31.6)	-	67.3	4.8	15.3	48.3
		+	27.9	<1	36.4	<1
B1	(54.8)	-	39.2	<1	47.4	10.1
		+	56.4	4.4	3.0	39.5
Anti-DR	(61.3)	-	36.5	<1	40.7	<1
		+	58.3	5.0	1.7	57.6
Leu M3	(5.6)	-	90.0	4.5	47.6	47.8
		+	4.4	1.1	1.3	3.3

* Control:non-related Mab: 0.7

TABLE V

PERCENTAGE OF TONSIL CELLS BOUND BY MABS

TONSILS*			Mab 25		Mab 135	
			-	+	-	+
TOTAL CELLS			9.4		33.2	
Leu 1	(40.3)	-	53.4	9.4	26.2	27.6
		+	37.2	<1	46.2	<1
B1	(41.6)	-	50.1	4.4	60.4	9.7
		+	39.2	6.3	3.1	26.8
Anti-DR	(43.8)	-	50.7	<1	62.3	<1
		+	41.8	7.5	2.5	35.2
Leu M3	(2.0)	-	90.5	7.6	65.1	33.9
		+	<1	1.9	<1	1.0

* Control:non-related Mab: 0.9

TABLE VI

PERCENTAGE OF PERIPHERAL BLOOD MONONUCLEAR CELLS BOUND BY MABS

PBL*			Mab 25		Mab 135	
			-	+	-	+
TOTAL CELLS			4.0		7.3	
Leu 1	(67.5)	-	28.6	5.7	28.1	7.3
		+	65.7	<1	64.6	<1
B1	(8.1)	-	90.3	<1	87.6	5.1
		+	6.6	3.1	1.6	5.7
Anti-DR	(20.1)	-	76.5	<1	78.5	<1
		+	19.9	3.6	13.4	8.1
Leu M3	(12.0)	-	84.9	4.3	80.1	8.2
		+	9.7	1.1	9.5	2.2

* Control:non-related Mab: 0.7

b) Mab 25

This antibody binds to approximately 4% of blood mononuclear cells, 4.8% spleen mononuclear cells and 9.4% tonsil mononuclear cells. It does not bind to T cells (Leu 1 positive). It preferentially binds to B cells (B1 positive) but not all of them. It also stains some non-T non-B lymphocytes, some Leu $M3^+$ cells (specific for monocytes) and some Leu $M3^-$ cells.

c) Mab 135

This antibody binds to approximately 7.3% blood mononuclear cells, 52.2% spleen mononuclear cells and 33.2% tonsil mononuclear cells. It does not bind to T cells (Leu 1 positive). It binds to almost all B cells (B1 positive) and to a fraction of the monocytes (Leu M3 positive). It binds to almost all the DR positive spleen and tonsil mononuclear cells but only to a fraction of blood DR positive cells.

4. Binding pattern to activated PBL

a) Technical aspects

Isolated peripheral blood mononuclear cells are resuspended at 5×10^5 cells/ml into culture medium (RPMI 1640 + glutamine 2 mM + penicillin (100 U/ml) + streptomycin (100 µg/ml) + 10% Fetal Calf Serum) in the presence of the following mitogens:

Phytohemagglutinin 1% (PHA) (GIBCO, Grand Island); Concanavalin A 10 µg/ml (Con A) (Pharmacia, Uppsala, Sweden); Staphylococcus aureus strain Cowan I, 0.01% (SAC) (Pansorbin, Calbiochem, La Jolla, Ca.); Pokeweed Mitogen, 1/100 dilution (PWM) (GIBCO, Grand Island, NY). Cells were harvested five days later, washed three times and stained with the Mabs as described earlier. Data are given in Table VII.

TABLE VII

BINDING OF MABS 25 AND 135 TO PBL
(PERIPHERAL BLOOD MONONUCLEAR CELLS)
STIMULATED FOR 5 DAYS WITH VARIOUS MITOGENS

	CONTROLS	MAB 25	MAB 135	
CON A	3.6%	2.3%	42.8%	
PHA	5.9%	5.9%	49.3%	EXPT 1
PWM	1.1%	4.2%	28.8%	
COWAN	7.7%	21.0%	23.8%	
CON A	ND	ND	ND	
PHA	4.7%	16.4%	52.2%	EXPT 2
PWM	0.8%	1.4%	42.5%	
COWAN	4.1%	3.2%	20.5%	

b) Mab 25

Its expression is only slightly or very slightly increased upon mitogenic activation.

c) Mab 135

The percentage of cells reacting with Mab 135 is increased upon mitogenic stimulation.

5. Immunoprecipitation analysis

a) Technical aspects

a1) Labelling of cells:

(50 to 100) x 10^6 cells are first washed twice with PBS and then resuspended in 2 ml PBS. To this

suspension the following reagents are added: 0.5-1 mCi $Na^{125}I$ (Amersham,). 100 µl lactoperoxidase at 5 mg per ml (Calbiochem) and 50 µl H_2O_2 (0.03% in water). The suspension is agitated for 3 minutes at room temperature. Another 100 µl lactoperoxidase and 50 µl H_2O_2 are added. The suspension is again agitated for 3 minutes at room temperature. This step is repeated once again and the cells are finally washed with PBS containing 5mM KI. The labelled cells are then resuspended at 5 x 10^6 per ml in PBS containing 0.5% Nonidet P-40, 1mM phenylmethylsulfonyl fluoride (PMSF), 0.01 M benzamidine hydrochloride, 0.05M aminocaproic acid, 20mM iodoacetamide, 10 µg/ml leupeptin, 1 µg/ml pepstatin and 100 µg/ml soybean trypsin inhibitor, and are incubated at 0°C for 20 minutes. The cells are centrifuged at 3000X g for 20 minutes and the supernatant is stored at -70°C. The lysate is clarified by centrifugation at 100,000X g for 30 minutes.

a2) Immunoprecipitation of membrane antigens:

The lysates are first precleared overnight with an ascite containing a non-related monoclonal antibody. Then, a rabbit anti-mouse immunoglobulin coupled to formalinized Staphylococcus aureus strain Cowan is added. After centrifugation the lysate is ready for the immunoprecipitation, which is performed as in the preclearing step except that the non-related Mab is replaced by the antibody to be assayed.

b) Mab 25:

It specifically precipitates a 42-44 Kd molecule which corresponds to the established data for $Fc_\varepsilon R_{II}$ using affinity chromatography on immobilized IgE.

c) Mab 135:

It immunoprecipitates three different molecular entities: a 44-46 Kd molecule (which may be different from that precipitated by Mab 25) and also molecules of about 35 Kd and about 30 Kd.

6) Determination of the affinity (Ka) of Mab 25

The affinity of Mab 25 for its specific cell surface antigen was performed according to Trucco and de Petris (Immunological Methods, Vol III, Ed: I. Lefkovits, B. Pernis, Academic press, 1981, pp. 1-26).

a. Technical aspect

a1. Iodination of Mab 25

50 µg of Mab 25 in 100 µl of PBS are mixed with 1 mCi $Na^{125}I$ (5 µl) and chloramine T (5 µl at 0.8 mg per ml in PBS). After a 10 minutes' incubation period at room temperature under gentle shaking, 5 µl sodium bisulphite (1 mg/ml in PBS) and 250 µl of ovalbumin (5% in PBS) are added to stop the reaction. The solution is passed over a PD10 column (Pharmacia, Sweden) pre-equilibrated with PBS containing 0.1% Tween 20 (Merck, Germany) and 1% bovine serum albumin. This step permits recovery of the iodinated antibody without free iodine.

a2. Determination of the affinity and of the number of receptors

Increasing concentrations (0.1 µg to 4µg) of the iodinated Mab 25 are added to 10^6 RPMI 8866 cells in a final volume of 1 ml of RPMI 1640 medium containing 2% BSA. For antibody concentrations of 0.3, 0.5 and 1 µg/ml,

additional tubes are prepared in which non-labelled Mab 25 is added in a 500-fold excess in order to determine non-specific antibody binding. Binding lasts 3 hours at 4°C. Cells are finally washed three times and the radioactivity in the final cell pellet is determined in a gamma counter. The data are finally plotted using the standard Scatchard analysis.

b. Affinity and number of receptors of Mab 25

The affinity of Mab 25 for the RPMI 8866 $Fc_\varepsilon R_{II}$ is $1.2 \times 10^8 M^{-1}$ and 435,000 receptors are identified.

Kit for detection of cellular $Fc_\varepsilon R_{II}$

I) Detection of $Fc_\varepsilon R_{II}$ on peripheral blood cells.

Conventional IgE rosette assays suggest that atopic patients display increased expression of $Fc_\varepsilon R_{II}$ on their blood mononuclear cells. Mab 25 permits a much more accurate determination of $Fc_\varepsilon R_{II}$ expression and is useful in this type of clinical investigation. Determining the expression of $Fc_\varepsilon R_{II}$ on blood mononuclear cells is useful in a number of clinical situations related to atopy. The Mab 25 can be made available in a lyophilized form either unlabelled in itself but accompanied by an appropriately labelled second stage antimouse Ig antibody or labelled with either FITC or biotin. To detect biotin as label, FITC- or phycoerythrin-labelled avidin can be used.

II) Detection of $Fc_\varepsilon R_{II}$ on human T cell clones.

It has been demonstrated in rodents that $Fc_\varepsilon R_{II}$-positive T cells play a major role in the regulation of IgE synthesis. Mab 25 is presently used to

establish human $Fc_\varepsilon R_{II}$-positive IL_2-dependent human T cell clones. Such clones should prove highly valuable tools to elucidate the regulation of IgE production in humans.

Kit for detection of soluble $Fc_\varepsilon R_{II}$ by competition assay

Mab 25 is coupled to beads or wells of microtiter plates. Samples to be assayed for the presence of soluble $Fc_\varepsilon R_{II}$ or a standard preparation of $Fc_\varepsilon R_{II}$ are first incubated with a known amount of labelled soluble $Fc_\varepsilon R_{II}$. After incubation they are transferred to and allowed to react with the insolubilized antibody on the beads or in the wells. The labelled soluble $Fc_\varepsilon R_{II}$ can be either i) a ^{125}I iodinated recombinant $Fc_\varepsilon R_{II}$; ii) an artificial fusion product between $Fc_\varepsilon R_{II}$ and an enzyme e.g. E. coli β-galactosidase; or iii) a biotinylated $Fc_\varepsilon R_{II}$. In case i) the reaction will be monitored through gamma counting; in case ii) the reaction will be monitored through optical measurement of degradation substrates specific for the enzyme (e.g. the reaction will be monitored by β galactosidase); in case iii) optical measurement of degradation substrates specific for the enzyme which was coupled to the biotin-specific avidin. In any case the presence of soluble $Fc_\varepsilon R_{II}$ in the sample to be assayed will induce a decrease in the observed signal.

CLAIMS

1. Monoclonal antibodies inhibiting binding of soluble IgE to human lymphocytes.

2. Monoclonal antibodies to the human lymphocyte IgE receptors.

3. Antibodies according to claim 1 or claim 2 that are of immunoglobulin subclass IgG_1, in particular those designated Mab 25 and Mab 135 herein.

4. Monoclonal hybridomas capable of producing monoclonal antibodies inhibiting binding of soluble IgE to human lymphocytes.

5. Monoclonal hybridomas capable of producing monoclonal antibodies to the human lymphocyte IgE receptors.

6. The monoclonal hybridomas designated herein as 9P25 and 9P135 D6/5.

7. Use of a monoclonal antibody as claimed in any of claims 1 to 3 to assay cells bearing the human $Fc_\varepsilon R_{II}$ receptors.

8. Use of Mab 25 to immunoprecipitate a 42-44 Kd membrane molecule which is the $Fc_\varepsilon R_{II}$.

9. Use of Mab 135 to immunoprecipitate three membrane molecules with molecular weights in the regions of 44-46, 35 and 30 Kd.

10. Use of Mab 25 in a competition immunoassay for the detection of soluble $Fc_\varepsilon R_{II}$ and IgE BF.

11. Use of Mab 25 to establish human IL_2-dependent-T cell lines bearing the $Fc_\varepsilon R_{II}$.